In vitro Antimicrobial Activity and Phytochemical Screening of *Ammi visnaga* Against Enteric Microorganisms

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ABSTRACT

This study was carried out to evaluate the antimicrobial activity and phytochemical screening of *Ammi visnaga* (Khella) against standard and clinical isolates from patients suffering from gastrointestinal tract infections. A total of fifty stool samples were collected from different hospitals, cultured in different media, isolated and identified. The isolated organisms were as follows: *Escherichia coli* (32%), *Salmonella enterica (typhi) (*18%), *Proteus vulgaris (*12%), *Staphylococcus aureus* (14%), *Klebsiella pneumonia* (10%) and *Shigella dysentery* (14%). The plant was extracted with two solvents (ethanol and hexane), ethanol extracts showed higher activity than hexane extracts.

Effect of Ciprofloxacin and Nalidixic acid antibiotics were also tested gainst all the isolated bacteria in addition to *Candida albicans* (standard). Ciprofloxacin showed high activity to all the strains while Nalidixic acid gave low activity. This study proved that *Ammi visnaga* (100mg/ml) has inhibited all the bacteria and fungi recruited in this study, while Ciprofloxacin is the antibiotic of choice for all these tested microorganisms.

metabolite contents e.g (Alkaloid ++, Flavonoid ++). Drug industries should recruit this

plant (*Ammi visnaga)* since its active against gastrointestinal pathogens to skip the ongoing resistance of antibiotics against microorganisms

مستخلص

لقد أجريت هذه الدراسة لتقييم النشاط الضد مكروبي والمسح الكيميائي النباتي لنبات الخلة (*Ammi visnaga*) ضد عزلات قياسية وسريرية من مرضى يعانون من إصابات الجهاز الهضمي. خمسون (50) عينة اخذت من البراز وزرعت في منابت مختلفة، تم تنقيتها ومعرفة أنواعها وكانت كالاتي: أي كولاي (*E.coli*) (32%)، سامونيلا انتيريكا (تايفي) *Samonella enterica* (18%)، بروتاس فلجارس *Proteus vulgaris* (12%) العنقودية الذهبية استافيلوكوكس *Staphylococcus* (14%)، كلبسيلا نيوموني *Klebsiella pneumonia* (10%) وشيقيلا ديسنتري *Shigella dysentery* (14%). لقد تم استخلاص النبات بواسطة مذيبين هما الايثانول ethanol والهيكسين Hexane. مستخلص الايثانول أعطى نشاطاً اكبر من مستخلص الهيكسين. ولقد تم ايضاً اختبار تأثير المضادات الحيوية سيبروفلوكسين Ciprofloxcin وحامض ناليديكسيك Nalidxic acid ضد كل العزلات البكتيرية، وشمل الاختبار أيضا فطر كانديدا البيكانس القياسية *Candida albicans* (Standard). اعطى سيبروفلوكسين تأثيراً أعلى على كل السلالات البكتيرية بينما لحامض الناليديكسيل تأثير ضعيف. لقد اثبتت هذه الدراسة مستخلص نبات الخلة (100 mg/ml) أثبط نمو كل سلالات البكتيريا والفطر كانديدا، وأن سيروفلوكساسين هو المضاد الحيوي الأفضل بالنسبة لكل الميكروبات المختبرة. اما بالنسبة للفحص النباتي الكيميائي لنبات الخلة فقد اعطى مركبات أيض ثانوية مثل (الكالويدات ++Alkaloid وفلافونويدات ++Flavonoids). ان صناعة العقاقير يمكنها استيعاب نبات الخلة (*Ammi visnaga*) والاستفادة منه نسبة لنشاطه ضد امراض الجهاز الهضمي لتفادي مقاومة الميكروبات للمضادات الحيوية.

1. INTRODUCTION

For thousands of years, natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other modern drugs. The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. It is estimated that local communities have used about 10% of all flowering plants on Earth to treat various infections, although only 1% have gained recognition by modern scientists. Owing to their popular use as remedies for many infectious diseases, researches for plants containing antimicrobial substances are frequent. (Betoni *et al*., 2006).

Ammi visnaga belongs to Family: Apiaceae. Botanical Description: Annual herb, reaching 1m in height at maturity has numerous branches. Leaves compound alternate, pinnately lobed, entire margins. Inflorescence compound amble about 8cm, flowers bisexual white to greenish. This plant is cultivated in many places especially in North Africa. The fruits of *Ammi visnaga* were used in the treatment of mild angina symptoms and as supportive treatment for mild obstruction of the respiratory tract in asthma, bronchial asthma or spastic bronchitis, and postoperative treatment of conditions associated with the presence of urinary calculi. Also it is used for treatment of gastrointestinal cramps and painful menstruation, diabetes and kidney stones. (WHO, 2007).

2. MATERIALS AND METHODS

2.1 Collection of specimens

Stool specimens were collected from Bashaer Hospital and IUA clinic during June 2015 to November 2016 and brought to the lab within 1 hour and cultured in already prepared media. Each specimen was labeled by code number before examination.

50 samples were collected randomly from different hospitals in Khartoum. Identifications of clinical isolates were carried out by inoculation of specimens into basic, differential and selective media (Manitol salt agar, MacConkey agar, EMB agar, XLD agar). Then the purified isolates were identified by microscopic xamination using gram staining technique, cultural characters and biochemical tests (KIA, Citrate, Urease, Indole), and then stored in a refrigerator until used.

2.2 Collection and preparation of plant samples

The selected plant for this work was Ammi visnaga as shown in (Table 1). It was obtained from Altaiman Spice Store at Ma'moura in Khartoum. The dried seed and pericarp samples were separately crushed to a powder by using mortar and pestle.

Table 1. Scientific name of the plant and part used

Family	**Scientific name**	***Local name***	***Part used***
Apiaceae	*Ammi visnage*	Khella	Seeds

2.3 Preparation of crude extract of plant materials

Fifty grams from each plant powder were extracted into 300ml of ethanol and hexane separately using Soxhlet Apparatus and the process was repeated several times depending on the amount of extract obtained from the process. Resulting extraction in the solvent was evaporated and concentrated using the rotary evaporator at 80°C. The highest concentration was 100mg/ml. Methanol was used as dissolving solvent for all the extracts. (Abeysinghe, 2010).

2.4 Testing of the Extracts for Antibacterial Activity

The cup-plate agar diffusion method was adopted according to Kavanagh (1972). To assess the antibacterial activity of the prepared extracts. 0.4 ml of standardized bacterial stock suspensions (108-109) colony- forming units (cfu) per ml was thoroughly mixed with 40 ml of sterile nutrient agar. Twenty ml of the inoculated nutrient agar were distributed into sterile Petri dishes. The agar plate were then left to set and in each of these plates 3 cups, 10 mm in diameter, were cut using a sterile cork borer No. 4 and the agar discs were removed. Alternate cups were filled with 0.1ml of each of the extracts using micro titer-pipette and were allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37ºC for 18 hours. Two replicates were carried out for each extract against each of the test organism. Simultaneously, pouring of the respective solvents instead of extracts were carried out as controls. After incubation the diameters of the

results and the growth inhibition zones were measured, averaged and the mean values were tabulated.

2.5 Testing of anti-fungal activity

The same methods used for the bacteria were adopted for fungi. Instead of nutrient agar, Sabouraud-dextrose agar was used. The media was inoculated and incubated at 25ºC overnight for *Aspergillus niger* and *Candida albicans.*

2.6 Minimum inhibitory concentration (MIC)

Plates were prepared in the series of increasing concentrations of the plant extraction in the following order 100, 50, 25 and 12.5mg/ml. The bottom of each plate was marked off into 4 wells. The viable organisms tested were grown in broth overnight to contain 108 CFU / ml. Loop-full of diluted culture was spotted on the surface of each plate and then incubated at 37 ºC for 24 hours. The (MIC) is the least concentration of antimicrobial agent that completely inhibits the growth. Results were reported as the MIC in mg/ml. The viable organisms tested were grown in broth overnight to contain 108 CFU / ml.

2.7 Sensitivity Test of Ciprofloxacin and Nalidixic acid

Sensitivity test was performed by modified Kirby – bauer (1972) disc diffusion method. Well isolated colonies of similar appearance were touched by using sterile loop and emulsified in 2ml of sterile normal saline; the turbidity of the suspension was matched to turbidity of 0.5 McFarland standards. Then sterile swab was immersed in the suspension and the excess was removed by pressing and rotating swab against the side of the test tube

above the level of the suspension. The plate of Muller – Hinton agar was inoculated by swab, using sterile forceps the antibiotic discs were placed on the inoculated plates and within 30 seconds of application the plates were incubated aerobically at 37oC for 24 hours. Each zone of inhibition was measured in mm and interpreted by chart, and then the organism was reported as sensitive or as resistant.

2.8 Phytochemical screening

Phytochemical screening of the extract was carried out to identify the constituents, using standard phytochemical methods as described in the following. The tests were carried to ensure the presence of tannins, alkaloids, saponins, cardiac glycosides, steroids, flavonoids and phenols. These tests were:

2.8.1 Detection of flavonoids

Lead acetate Test: 1ml of extract was treated with a few drops of 10% lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.

2.8.2 Detection of alkaloids

The extracts were dissolved individually in a dilute hydrochloric acid and filtered.

Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicated the presence of alkaloids.

2.8.3 Detection of glycosides

0.5 ml of extract was added 2.0 ml of glacial acetic acid containing one drop of FeCl3 Solution. This was then added with 1.0 ml of concentrated H2SO4. A brown ring obtained at the interface indicated the presence of glycosides.

2.8.4 Detection of saponins

Froth Test: 0.5ml Extracts were diluted with 5ml of distilled water. €The solution was shaken vigorously and observed for the stable persistent froth. Formation of 1 cm layer of foam indicated the presence of saponins.

2.8.5 Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of 10% ferric chloride solution. Formation of bluish black color indicated the presence of phenols.

2.8.6 Detection of tannins

0.5ml of the extract was boiled with 10ml of distilled water in a test tube and then, a few drops of 0.1% Ferric chloride solution was added and the reaction mixture was observed for blue or greenish black color change.

2.8.7 Detection of terpenoids / steroids (Salkowski test)

0.5ml of each of the extracts was added, 2ml of chloroform and then 3ml of the concentrated H2SO4 were carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids / steroids. (Audu, 2007), (Obasi, 2010), (Roopashree, 2008),

3. RESULTS AND DISCUSSION

In the present study *Ammi visnaga* seeds were extracted and screened for its antimicrobial activity using two solvents (Ethanol and Hexane). In Table 2. extracts showed inhibitory results against the clinical microorganisms used in this study which may be due to the high content of secondary metabolites e.g. Alkaloids, Flavonoids and Tannins etc. Hexane extracts of *A. visnaga* were less active than the ethanol extracts. *A. visnaga* (conc. 100 mg/ml) is effective against all Gram-positive and Gram-negative bacteria under study.

Table 2. Antibacterial activity of *A. visnaga* against the clinical isolates and standard

Microorganism	***Ammi visnaga*** (Khella)	
	Hexane	**Ethanol**
Staphylococcus aureus	18	24
Candida albicans	20	22
E. coli	17	21
Klebsiella pneumonia	0	20
Pseudomonas aeruginosa	0	18

Ammi visnaga ethanol extract showed good results. Inhibition zones against clinical isolates *Staphylococcus aureus, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* 24, 21, 20 and 18mm respectively. Table 3. shows that Ciprofloxacin was the most active antibiotic used and as the manufacturer of this antibiotic noted, Ciprofloxacin is the antibiotic of choice for all enterobacteriaceae. Inhibition zones against *E. coli* and *S. aureus* were 30 and 35mm, respectively, while Nalidixic acid showed very poor results against all types of bacteria in this study. The inhibition zones against *E. coli* and *S. aureus* were 19 and 7mm, respectively.

Table 3. Mean of MIC of *Ammi visnaga* (mm) and inhibition zones (mm) of the reference drugs (Ciprofloxacin and Nalidixic acid) against clinical isolates

Tested Microorganisms	**Solvents**	**Conc. of extracts (mg/ml)**	**Inhibition zones of *A. visnaga***	**Ciprofloxacin (5µg/disc)**	**Nalidixic acid (30µg/disc)**
Staphylococcus aureus	Ethanol	25	12	35	7
	Hexane	50	13		
Candida albicans	Ethanol	12.5	18	32	10
	Hexane	50	16		
Escherichia Coli	Ethanol	12.5	11	30	19
	Hexane	50	12		

Klebsiella pneumonia	Ethanol Hexane	12.5 50	15 12	12	0
Proteus vulgaris	Ethanol Hexane	12.5 50	13 12	28	13
Salmonella typhi	Ethanol Hexane	12.5 50	12 14	33	15
Shigella dysentery	Ethanol Hexane	12.5 50	13 12	30	10

The present study is in agreement also with the results of Ghareeb *et al.,* (2011) who concluded that ethanol extracts of *Ammi visnaga* inhibited the growth of *S. aureus, E.coli, K. pneumoniae* and *Candida albicans* with an MIC of 12.5mg/ml as in Table 3. The study is in agreement with the findings of Shadia (2000) who concluded that *A. visnaga* has effective activity against gram positive and gram negative bacteria.
The study is also in agreement with the findings concluded that *S. typhi, K. pneumonia* and *S. dysenteriae* were susceptible to Ciprofloxacin (Mamuye *et al.,* 2015). The results in Table 4. showed that *A. visnaga* seeds have the tested secondary metabolites but in different concentrations (Alkaloids, Flavonoids, Glycosides, Saponins, Tanins, Phenols and Terbinoids).

Table 4. Phytochemical screening of *Ammi visnaga*

Plant Extract	**Solvent**	**Flav**	**Sap**	**Alk.**	**Glycosides**	**Phenols**	**Tan**	**Terb**
Ammi visnaga	Hexane	+	+	+	+	+	+	+
	Ethanol	++	+	++	++	+ +	+	+

Key: Flav: Flavonoids Sap: Saponins Alk: Alkaloids

The present study is contrary to the results of Amin *et al.,* (2015) who concluded that *A.visnaga* has neither alkaloids nor saponins, while this study proved the presence of these two secondary metabolites in the plant.

4. CONCLUSION

In present study fifty specimens were collected aseptically from different patients. Fifty clinical isolates of 6 genera were isolated and identified, they were: *Staph. aureus, E. coli, Klebsiella pneumoniae, Proteus vulgaris* and *Salmonella enterica (typhi).* All the isolated bacteria mentioned above were treated with *Ammi visnaga* extracts and showed inhibition zones. The phytochemical screening tests of *Ammi visnaga* extracts has verified the presence of secondary metabolites (Alkaloids, Flavonoids, Saponins, Glycosides, Phenols, Tannins and Terbenoids).

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